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Bioactivity-guided isolation of anticancer compounds from *Euphorbia lathyris*

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1. Introduction

Based on the number of cases reported, cancer is considered as a fatal disease in the world.[1, 2] However, the method of cancer therapy is very limited. Current primary treatment for cancer is chemotherapy, but serious side effects have been reported.[3] Thus, a continued effort for discovery of novel anticancer compounds is needed. *Euphorbia lathyris*, a potent and valuable source of traditional Chinese medicine (TCM) which has been used hydropsy, ascites, coprostasis, anuresis, amenorrhea, stasis, terminal schistosomiasis, scabies and snakebite.[4] Previous studies of the anticancer activity of *Euphorbia lathyris* showed that bioactive components had mainly focused on water or alcohol extracts. Recently, numerous researches demonstrated that the ethyl acetate extracts of *Euphorbia lathyris* have more potent and broad-spectrum anticancer activity than its hot water and ethanol extracts.[5] The anticancer constituents of the ethyl acetate extracts from *Euphorbia lathyris* were not investigated earlier. The aim of the present work was the isolation and identification of the anticancer compounds from the ethyl acetate extracts of *Euphorbia lathyris* using bioactivity-guided separation method.

Searching for natural bioactive anticancer compounds requires appropriate bioassays. Recently, a new technological advance which is an electronic real-time cell analyzer (RTCA) makes it possible to obtain real time data on changes in cell growth,

morphology and cell death induced by drugs.[6] As the measurement is non-invasive and label free, the system can continuously monitor cells from the time when they are seeded. In addition, compared to MTT assay, RTCA provides precise information not available with other technologies after the addition of chemical compounds are recorded. The results are presented as a cellular index (CI), the more live cells that are attached to the sensor surface, the higher the CI value.[7, 8]

In previous work, separation and purification of compounds from *Euphorbia lathyris* mainliy relied on traditional methods of separation and purification, such as column chromatography, TLC, macroporous resin chromatography, and gel exclusion chromatography. Nevertheless, because of successive extractions with solvents, successive column isolation, low column efficiency, bad separation repeatability and limited column resolution, leading these fractions obtained with low pharmacology activity and poor purity.[9-13] Taking these problems into consideration, two-dimensional preparative HPLC, which involves coupling two independent mechanisms of separation, is regarded as more selective and having more resolving power than one-dimensional chromatography; it is, therefore, more useful for separation of complex samples.[14]

Two-dimensional RP–RPLC is a practical separation method and has been used for isolation of compounds from pharmaceutical samples.[15] Two-dimensional HILIC coupled with RP liquid chromatography has been proved to be very useful for separation of the components of *Taxus baccata* L.[16] Although these preparative 2D HPLC systems enable reasonable separation of compounds with a wide range of polarity and can, to some extent, solve the problems of peak capacity and separation selectivity, there are still specific deficiencies in comprehensive characterization of weakly polar TCM extracts, because of limitations of the modes of separation and the complexity of the samples. Thus, a specialized 2D HPLC method for preparative separation of weakly polar extracts of natural products is urgently needed.

In addition, two-dimensional HPLC can be performed either off-line or on-line. In this study, off-line 2D-LC analysis of TCMs is seemingly time-consuming method,

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but it provides valuable information for the separation of active compounds in one-dimensional HPLC or even multi-dimensional HPLC analysis. Moreover, considering that the off-line mode has no restrictions on the screening of the bioactive components using RTCA, so the bioactive fractions eluted from the first dimension could be prepared and analyzed with enough time.[17] Therefore, the off-line mode was selected to maximize resolving power of two dimensions.

The purpose of the work discussed in this paper was to develop a simple method, with *Euphorbia lathyris* example, based on the use of a novel nonaqueous 2D preparative high-performance liquid chromatography (PHPLC), the real-time cell analyzer (RTCA), and X-ray single crystal diffraction (XRSCD), for efficient isolation and identification of anticancer compounds.

2 Results and Discussion

2.1 Establishment of Bioactivity-guided Off-Line 2D Chromatography System

In bioassay-guided isolation, the steps of the procedure (separation, fractionation, and purification) are systematically followed by bioassay, i.e. the next step of the process is based on a bioassay result. For this reason, rapid, reliable and relatively simple biological detection is needed. RTCA, which enables rapid measurement of the dynamic biological response of living cells in the presence of compounds which have cell growth-inhibiting or promoting properties, meets these demands.

For the sake of constructing a specialized 2D HPLC preparative separation method, several columns were tested. In consideration of the weak polarity of the components in our samples, nonaqueous column seemed to be the only option for the preparative separation. Moreover, to achieve better separations, selection of mobile phases of appropriate composition is also very important.

The main objective of the first-dimensional preparation is to simplify complex samples into fractions. The bare silica column is the most traditional nonaqueous normal-phase column for separation of weakly polar compound.[18] Mobile phases used in traditional normal phase chromatography consist of a non-polar solvent and small amounts of polar organic solvents.[19] In this work, several polar solvents mixed with a

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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

88 non-polar solvent (n-hexane) were investigated to test the results of NP separations.
89 When UV detection is used, the choice of mobile phase is limited. Because of
90 interference as a result of solvent absorption in the UV region, the combinations
91 n-hexane–acetone and n-hexane–ethyl acetate did not provide good baseline
92 separation. However, the results showed that a mixture of n-hexane and ethanol as
93 binary mobile phase for NP chromatography has such advantages as wide polarity
94 range, by changing the ratio of the two solvents, and relatively low environmental
95 toxicity. Thus, bare silica columns with a nonaqueous mobile phase were selected for
96 separation in the first dimension.

97 In normal phase (NP) systems, the following equation was found to adequately
98 describe the retention model[23]:

99 $$\log k = \log k' - m' \log \varphi \quad (1)$$

100 where φ was the volume fraction of the polar solvent, k' was the retention factor, and
101 m' was the stoichiometric coefficient characterizing the number of the molecules of
102 the strong solvent that were needed to displace one adsorbed molecule of the analyte.

103 There were three variables that affect the retention factor k, which are k', m', and
104 φ. When the analytes and mobile phase system (n-hexane/ethanol) were determined,
105 the retention factor k' could be tested and the k' became a constant. On the basis of the
106 condition that analytes and mobile phase system were both determined, if the
107 concentration of the sample solution and loading volume were invariable, the number
108 of the molecules of the strong solvent that were needed to displace one adsorbed
109 molecule of the analyte could also be determined, which means that the variable m'
110 became a constant. Hence, we defined, Eq. (1) could be simplified as:

111 $$\log k = \log K_1 - M_1 \log \varphi \quad (2)$$

112 Here K_1 and M_1 were two constant parameters and φ was the volume fraction of the
113 ethanol.

114 The main objective of the second-dimensional preparation is to provide
115 high-performance and rapid preparation for the fractions collected from the
116 first-dimensional preparation. High-performance separation benefits from the

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second-dimensional column whose retention mechanism is different as the first-dimensional column, it was possible to form an orthogonal separation system. Of all two-dimensional liquid chromatography methods, the coupling of normal phase liquid chromatography (NPLC) to reverse phase liquid chromatography (RPLC) holds most promise because of the orthogonal nature of the separation mechanisms.[15, 20, 21] A typical mobile phase for RPLC includes water-miscible polar organic solvents such as acetonitrile with a small amount of water.[6] Unfortunately, weakly polar samples are usually too strongly retain in RP LC and (or) are not sufficiently soluble in the aqueous mobile phases. HILIC is a complementary technique to both normal and reversed-phase chromatography for the separation of compounds.[18, 22] This problem can be solved by using Hydrophilic interaction chromatography (HILIC) amide column. However, amide column was previously usually used in aqueous HILIC mode.[23] In nonaqueous chromatography, amide column was only used for analysis.[24] No report on amide preparative column coupled with bare silica column used in nonaqueous 2D preparative chromatography could be found.

For HILIC mode, the retention model can be described by the following equation:

$$\log k = \alpha + m_1\varphi_1 - m_2\log\varphi_1 \quad (3)$$

The α is an empirical constant. The term $m_1\varphi_1$ was related to the interaction between solutes and solvents (RP effect). While $m_2\log\varphi_1$ was related to the direct analyte–stationary phase interaction (NP effect). Fig. S1 showed an example illustrating the effect of the mobile phase composition on the retention factors of caffeic acids. The U-shape curves got by increasing the concentration of water were similar, which provides evidence for the fact both HILIC and RP mode share the same separation mechanism. However, as the concentration of acetonitrile increasing, strong deviations start to take place. That showed the separation effects of the two different modes were opposite. This phenomenon goes for normal phase liquid chromatography. Therefore, when a double organic solvent system was employed on an amide column, the interaction energy between solutes and solvents became so

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4 146 weak. Then, the term $m_1\varphi_1$ could be approximately considered as a small and
5
6 147 nonignorable constant. Then, the variable φ_1 became φ that was the volume fraction of
7
8 148 the ethanol. So the NP term $-m_2\log\varphi_1$ became $-M_2\log\varphi$. Eq. (3) could be finally
9
10 149 simplified as the following equation:
11
12 150 $$\log k = K_2 - M_2\log\varphi \quad (4)$$
13
14 151 Among which K_2 was a constant, and M_2 was a constant that was a new
15
16 152 coefficient related to analyte–stationary phase interaction.
17
18 153 The two different parameters M_1 and M_2 indicated that the sensitivities of the
19
20 154 bare silica column and nonaqueous amide column against mobile phase changing
21
22 155 might be different. Additionally, the retention characters of the two columns might be
23
24 156 different too, due to their different intercepts K_1 and K_2. On the basis of the above
25
26 157 differences between the two columns, they would have selectivity characters that were
27
28 158 distinct enough to form an orthogonal 2D chromatography system.
29
30 159 **2.2 First Dimension Preparative HPLC**
31
32 160 The first dimensional preparation was carried on Agela bare silica prep column.
33
34 161 The sample injection volume for each run was 150 mL. One whole preparation
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36 162 procedure took 65 min including 10 min for column balance, 45 min for preparation
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38 163 and 10 min for column washing. The retention time of the components differed
39
40 164 significantly because of the complex composition of the extracts. Isocratic elution
41
42 165 fails to achieve satisfying separations. Hence, gradient elution was necessary in first
43
44 166 dimensional preparation. Linear gradient provided better separation performance for
45
46 167 the constituent with different retention times. Good separation performance can be
47
48 168 obtained in analytical HPLC (Fig. 1a). When using the same chromatographic
49
50 169 conditions in preparative HPLC, the similar retention times was obtained as analytical
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52 170 HPLC. Finally, analytical elution methods were used for the preparation. Preparation
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54 171 of the total 200 g extracts required 14 injections over 16 h. According to UV
55
56 172 absorption intensity, the fractions were collected to reduce the complexity of each
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58 173 fraction as much as possible. The cross in each fraction has been minimized because
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60 174 of good separation repeatability. As shown in (Fig. 1b), eleven fractions were

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collected in the first dimensional preparation. All fractions were analyzed by the RTCA using A549 and HEPG2 cells to evaluate their anticancer activities. As the results presented in Fig. 4 and Fig. 5, fractions 3, 5 and 6 showed higher bioactivity than the other fractions. Thus, they were worthy of further separation and purification by the second dimensional HPLC.

2.3 Analysis on Fractions 3, 5 and 6 on amide column and bare silica column

Fractions 3, 5 and 6 were analyzed on the YMC amide column to compare the separation selectivity with that on the Agela bare silica column. Good orthogonality was significant to achieve efficient preparation of compounds with high purity from the samples. For example, Fr. 3 was roughly a single peak on bare silica column and it might be regarded as a single compound in traditional technique where HPLC preparation was commonly used in the final step to yield pure compounds. In fact, on amide column, there were two peaks (Fig. 3). Moreover, Fr. 5-4 to Fr. 5-6 could be clearly recognized on amide column. However, on bare silica column their retention times were so close that it was impossible to obtain satisfactory resolution. For the coeluted compounds samples such as Fr. 6-7 (Fig. 3), it may be because of the presence of a variety of compounds of the same polarity and a relatively low concentration of minor compounds. But, multiple compounds with high purity can be prepared on the amide column. In view of its higher separation efficiency, better resolution and peak shape, the amide column was chosen as the second dimensional column for further separation. It not only improved the separation efficiency but also made a remarkable contribution to the purity of the compounds collected.

2.4 Preparative HPLC in the Second Dimension

In 2D LC, the second dimension LC is important in achieving higher separation efficiency. The second-dimensional separation was performed on an amide column. In order to purify the single compounds effectively, the separation condition was optimized for fractions 3, 5 and 6. In this study, preparative scale conditions were optimized in an analytical scale. For fraction 3, it was found that second-dimensional separation should be performed under isocratic conditions to avoid time-consuming

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4 204 reequilibration required for reconditioning in gradient elution. However, for fractions
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6 205 5 and 6, simple isocratic method was not enough. When using isocratic elution, high
7
8 206 concentration of ethanol at the beginning results in shorter retention times, even cause
9
10 207 changes of peaks order. Thus, gradient elution was necessary preparative separation in
11
12 208 the second dimension. After minor modification, the optimized conditions used for
13
14 209 analytical chromatography were used for preparative scale separations. The analytical
15
16 210 chromatogram in Fig. 2b showed similar patterns to the preparative chromatograms in
17
18 211 Fig. 3, which demonstrated the feasibility of the transformation from an analytical
19
20 212 scale to a preparative one. Moreover, it was also worthy of note that heart-cutting was
21
22 213 used as the repeated separation strategy to ensure the purity of the compounds. Good
23
24 214 separation resulted in several compounds were obtained and dried through rotary
25
26 215 evaporation at 60 ℃ in vacuum.
27
28 216 In bioassay experiments, each fraction was tested for *in vitro* anti-lung cancer
29
30 217 and anti-liver cancer activity, respectively (Fig. 4c - 4f and Fig. 5c- 5f). Among all the
31
32 218 fractions, the seven fractions (Fr. 3-4, 36 mg), (Fr. 3-5, 38 mg), (Fr. 5-4, 60.2 mg), (Fr.
33
34 219 5-6, 10.3 mg), (Fr. 6-3, 15.1 mg), (Fr. 6-7, 18.2 mg) and (Fr. 6-11, 16.8 mg) had
35
36 220 different degree of growth inhibition effect against A549 and HEPG2 cells at a
37
38 221 concentration of 50 μg·mL^{-1}. Among all fractions, Fr. 3-5 and Fr. 6-7 showed
39
40 222 significant anticancer activity against A549 and HEPG2 cells. Fr. 3-4, 5-4, 5-6 and
41
42 223 6-11 were inapparent active against HEPG2 cells. However, when tested in the A549
43
44 224 cells, the four fractions showed different degree of anticancer activity. Fr. 6-3
45
46 225 exhibited a good inhibition of cancer cells growth activity.
47
48 226 The purity of these compounds was checked by HPLC, which is shown in Fig. 6
49
50 227 and all compounds were more than 95 % purity. As described above, the seven
51
52 228 fractions were of high purity, which require structural identification.
53
54 229 In a word, the first-dimensional preparation efficiently simplified the sample and
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56 230 improved the separation on the second-dimensional preparation, so that multiple
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58 231 compounds with high purity can be prepared at the second-dimensional preparation.
59
60 232 **2.5 Structural identification of bioactive compounds**

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4 233 Compounds 1-7 were identified by 1D (^{1}H (400 MHz), ^{13}C (100 MHz) and
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6 234 DEPT-135) and 2D NMR spectroscopy. Complete assignment of all the proton and
7
8 235 carbon data was based on ^{1}H–^{1}H COSY, HSQC, and HMBC data. The results are
9
10 236 listed in ESI Table S1 and Table S2. Comparison of the ^{1}H and ^{13}C NMR spectra of
11
12 237 these compounds with the previous reports characterized their structures as *Euphorbia*
13
14 238 Factor L2 (1), *Euphorbia* Factor L1 (2), Glyceryl monooleate (3), *Euphorbia* Factor
15
16 239 L8 (4), *Euphorbia* Factor L3 (5), *Euphorbia* Factor L9 (6) and Esculetin (7). The
17
18 240 structures of these compounds were shown in Fig. 7.[25-27]
19
20 241 Compound 2 was *Euphorbia* Factor L1, a compound that had been previously
21
22 242 reported.[28] Since the ^{1}H and ^{13}C spectra showed severely overlapping signals in the
23
24 243 high field region, and given that the chemical shift values slightly differed from
25
26 244 reported data, we confirmed the structure with the aid of X-ray diffraction analysis.
27
28 245 According to X-ray single-crystal diffraction (XRSCD) data, chiral centers C-2, C-3,
29
30 246 C-6, C-9, and C-11 were assigned the S configuration and C-4, C-5, and C-15 were
31
32 247 assigned the R configuration. The structure of compound 2 determined by XRSCD,
33
34 248 with the atom numbering scheme, is shown in Fig. 8. Selected bond distances and
35
36 249 angles are listed in ESI Table S4 and Table S5. On the basis of these data the absolute
37
38 250 configuration of compound 2 was confirmed.
39
40 251 **3. Experimental**
41
42 252 **3.1 Apparatus**
43
44 253 An industrial prep-HPLC system consists of two prep-HPLC pumps, a UV detector
45
46 254 and a HPLC workstation (Jiangsu Hanbon Sci. & Tech. CO., Ltd, Huaian, China). The
47
48 255 semi-preparation HPLC system was carried on Agela CHEETAH MP 100 system,
49
50 256 which consists of two binary gradient pumps, a UV detector, a sample collector and
51
52 257 an Agela LC software (Bonna-Agela Technologies, Tianjin, China). Chromatographic
53
54 258 analysis was performed with a Hitachi HPLC system comprising two Hitachi L2130
55
56 259 pumps, a Hitachi L2400 UV diode-array detector, Hitachi L2200 autosampler, column
57
58 260 thermostat and a Hitachi Lachrom Elite HPLC workstation (Hitachi, Tokyo, Japan).
59
60 261 The NMR spectrum was measured by a Bruker DXR 400 NMR spectrometer with

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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

262 $CDCl_3$ and dimethyl sulfoxide (DMSO) as solvents (Bruker AVANCE III; Bruker,
263 Karlsruhe, Germany). The iCELLigence RTCA system was composed of three main
264 components: an iCELLigence RTCA analyzer, RTCA control unit (iPad with
265 integrated software) and a disposable E-Plate L8 (ACEA Biosciences, USA).

266 **3.2 Reagents**

267 The Industrial and prep-HPLC grade ethanol, *n*-hexane and ethyl acetate were
268 purchased from Concord Technology Co. Ltd. (Tianjin, China). HPLC grade solvents
269 were purchased from Honeywell (USA). DMSO was purchased from the
270 Sino-American Biotechnology Company of Beijing (Beijing, China). Minimum
271 essential medium (MEM) and F-12K medium was purchased from Life Technologies
272 (USA).

273 **3.3 Sample Preparation**

274 *Euophorbia lathyris* was purchased from Anguo medicinal materials market (Anguo,
275 Hebei Province, China). The plant material was identified by Professor Lijun Zhou
276 (Tianjin University). Five kilograms *Euophorbia lathyris* powders were obtained by
277 ultrafine comminution (SQW-25; San Qing Technology Company, Jinan, China) at
278 low temperature. Then, the powder was extracted three times by 40 L ethyl acetate for
279 3 h. After centrifugation, the supernatant was combined and concentrated by rotary
280 evaporation (RE-52AA; Yarong Biochemical Instruments, Shanghai, China) at 60 ℃
281 in a vacuum. The ethyl acetate extract was dissolved in a solvent of *n*-hexane–ethanol
282 (70:30, v/v). The solution was filtered through 0.45 μm membranes. The final
283 concentration of sample solution was 200 mg $\cdot$mL $^{-1}$.

284 **3.4 Analytical HPLC**

285 **3.4.1 1D-HPLC analysis** The ethyl acetate extracts were chromatographed on an
286 Agela bare silica column (4.6 × 250 mm, 10 μm, 100 Å). The injection volume was 10
287 μL. The mobile phase was a gradient prepared from *n*-hexane (solvent A) and ethanol
288 (solvent B); the gradient program (v/v) was: 5 min 100 % solvent A, 40 min 0-13 %
289 solvent B, 10 min 100 % solvent B; the flow rate was 0.6 mL $\cdot$min^{-1}. HPLC analysis
290 was performed at room temperature and monitored at 210 nm.

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2
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4 291 **3.4.2 2D-HPLC analysis** Analysis of the bioactive fraction was performed at
5
6 292 room temperature by HPLC on YMC amide and Agela bare silica column (4.6 × 250
7
8 293 mm, 10 μm, 100 Å) with *n*-hexane (solvent A) and ethanol (solvent B). Different
9
10 294 elution conditions were adopted to analysis fractions collected from the first
11
12 295 dimensional separation. The chromatographic conditions for fraction 3 was 5 %
13
14 296 solvent B for 65 min, for fraction 5 was 0-20 % solvent B for 65 min, for fraction 6
15
16 297 was 0-25 % solvent B for 65 min. The injection volume was respectively 10 μL. The
17
18 298 flow rate was 0.6 mL $\cdot$min^{-1}. The chromatographic data was collected at 210 nm.
19
20 299 **3.5 Preparative HPLC**
21
22 300 **3.5.1 1D-HPLC Preparation** The first dimensional preparation was performed
23
24 301 on an Agela bare silica prep column (150 × 250 mm, 10 μm, 100 Å). The mobile
25
26 302 phase A was *n*-hexane and mobile phase B was ethanol. The linear gradient elution
27
28 303 steps were as followed: 0-5 min 100 % A, 5-45 min 0-13 % B, 45-55 min 100 % B.
29
30 304 The flow rate was 600 mL $\cdot$min^{-1}. The sample concentration was 200 mg $\cdot$mL^{-1}. The
31
32 305 injection volume was 150 mL. Chromatography was recorded at 210 nm. Each
33
34 306 fraction was dried under vacuum in a desiccator and stored under dry conditions for
35
36 307 further separation and purification.
37
38 308 **3.5.2 2D-HPLC Preparation** The second dimensional purification was
39
40 309 performed on a YMC amide column (10 × 250 mm, 10 μm, 100 Å). The mobile phase
41
42 310 A was *n*-hexane and mobile phase B was ethanol. Fractions collected from the first
43
44 311 dimensional separation were further purified on amide column to obtain single
45
46 312 compounds. Different elution conditions were adopted to separate fractions collected
47
48 313 from the first dimensional preparation. The elution procedure for fraction 3 was 5 % B
49
50 314 for 65 min, for fraction 5 was 0-20 % B for 65 min, for fraction 6 was 0-25 % B for
51
52 315 65 min. The sample concentration was 200 mg $\cdot$mL^{-1}. The injection volume was 50 μL.
53
54 316 The flow rate was 3 mL $\cdot$min^{-1}. The chromatographic data were collected at 210 nm.
55
56 317 **3.6 Bioassays**
57
58 318 The anticancer effect of fractions was evaluated by use of the iCELLigence RTCA
59
60 319 against A549 cells and HEPG2 cells. The iCELLigence RTCA system consists of 8

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1 2 3
4 320 well E-Plates (E-Plates 8) engaged on stations where the cell index (CI) reading was
5
6 321 recorded simultaneously. 150 μL MEM medium was added to each well of the
7
8 322 E-plates 8 for A549 cells and 150 μL F-12K medium for the HEPG2 cells,
9
10 323 respectively. Then, the plates were placed in the iCELLigence RTCA system and
11
12 324 scanned for 2 min at 37 ℃ and 5 % CO_2. The plates were incubated for 30 min to
13
14 325 obtain a stable noise signal. A549 cells (2×10^4/well) and HEPG2 cells (4×10^4/well)
15
16 326 were cultured in each well of the E-plates 8 respectively. Samples containing
17
18 327 untreated A549 and HEPG2 cells served as a control for the assay. Following
19
20 328 exposure to 50 $\mu g \cdot mL^{-1}$ samples, the cell index (CI) reading frequency was once per
21
22 329 minute until completion of experiment. The experiment was conducted for 48 h.
23
24 330 **3.7 Purity Analysis of Bioactive Compounds**
25
26 331 Purity test of bioactive compounds were performed on a YMC diol column (4.6×250
27
28 332 mm, 5 μm, 100 Å). The mobile phase A was *n*-hexane and mobile phase B was
29
30 333 ethanol. The flow rate was 1 $mL \cdot min^{-1}$. The injection volume was 10 μL. The elution
31
32 334 procedure for Fr. 3-4 and Fr. 3-5 was 2 % - 6 % B for 20 min, Fr. 5-4 and Fr. 5-6 was
33
34 335 0 % - 20 % B for 20 min, for Fr. 6-3 was 7 % B for 20 min, Fr. 6-7 was 0 % - 15 % B
35
36 336 for 25 min and Fr. 6-11 was 0 % - 25 % B for 25 min, respectively. Chromatographic
37
38 337 data were collected at 210 nm.
39
40 338 **3.8 NMR Spectrometry**
41
42 339 Structures of anticancer compounds were identified by one and two dimensional
43
44 340 nuclear magnetic resonance spectroscopy (1D and 2D NMR). NMR experiments were
45
46 341 performed with a Bruker AVANCE III NMR (400 MHz) spectrometer. Samples were
47
48 342 dissolved in $CDCl_3$ and DMSO, and chemical shifts were reported relative to the
49
50 343 resonance of TMS at $\delta = 0$ ppm as reference. Solution volume was concentrated to
51
52 344 400 μL and transferred to a 5 mm NMR test tube for analysis.
53
54 345 **3.9 X-ray single Crystal Diffraction**
55
56 346 Single crystal of compound 2 was performed by slow evaporation, at 4 ℃, of a
57
58 347 solution in n-hexane–tetrahydrofuran (1:1). A colorless single crystal ($0.25 \text{ mm} \times 0.20$
59
60 348 $\text{mm} \times 0.15$ mm) was selected and used for the X-ray diffraction experiment. X-ray

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diffraction data were collected on a Rigaku Raxis Rapid IP detector equipped with a graphite-monochromatic Mo-Kα radiation (k = 0.71073 Å) at 293(2) K. The orientation matrix and unit cell parameters were obtained from the setting angles of 25-centered reflection. The crystals were orthorhombic, space group P21212 with a = 8.2474(16) Å, b = 2.543(3)(16) Å, c = 29.019(6) Å, α = 90.00, β = 90.00, γ = 90.00, V = 3001.9(10) $Å^3$, Z = 4, Dcalc = 1.233 mg m^{-3}, and μ = 0.087 mm^{-1}. The diffraction intensities were collected by ω scanning (3.04-25.50). A total of 24,238/5,565 reflections were collected (-8 ≦ h ≦9, -15 ≦ k ≦15, -34 ≦ l ≦35). The crystallographic data and refinement details are summarized in Table S3. The crystal structure was solved by direct methods with SHELX-97 program and refined by full-matrix least-squares method.

4 Conclusions

On the basis of bioactivity-guided off-line isolation by 2D chromatography, seven anticancer compounds were isolated from an ethyl acetate extract of *Euphorbia lathyris*. A bare silica preparative column was used to obtain fractions from preparative separation in the first dimension. An amide preparative column used in nonaqueous mobile phases was employed to prepare compounds of high purity in the second dimensional preparation. Three fractions obtained from separation in the first dimension were analyzed on bare silica and amide columns. The results showed the good orthogonality of the 2D preparative separation. Benefitting from good orthogonality and optimized collection, seven bioactive compounds with > 95 % purity were obtained by 2D preparative chromatography.

A novel bioassay-guided separation system was established for the separation of complex weakly polar bioactive samples. In this system, a HILIC column used with a nonaqueous mobile phase had good orthogonality when coupled with a traditional normal-phase column. RTCA is a useful tool for study of TCM with anticancer potential and for monitoring the bioactivity of fractionated components and pure compounds, which can be identified by the use of intelligent biosensor techniques.

We believe this novel bioassay-guided off-line 2D HPLC method is extremely

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useful for preparative separation of weakly polar compounds and could lead to the discovery of more useful natural anticancer compounds.

Acknowledgments

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434 **Appendices**

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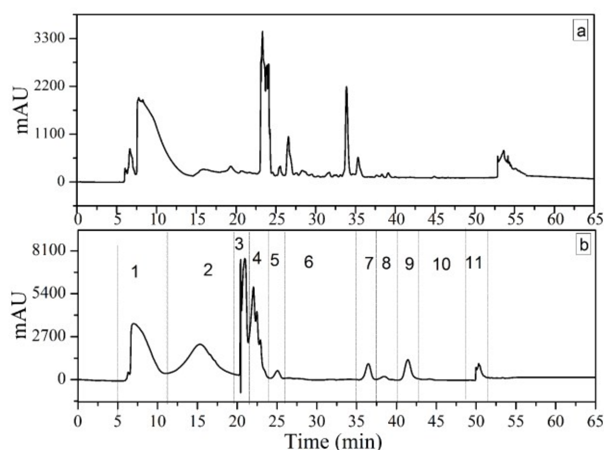
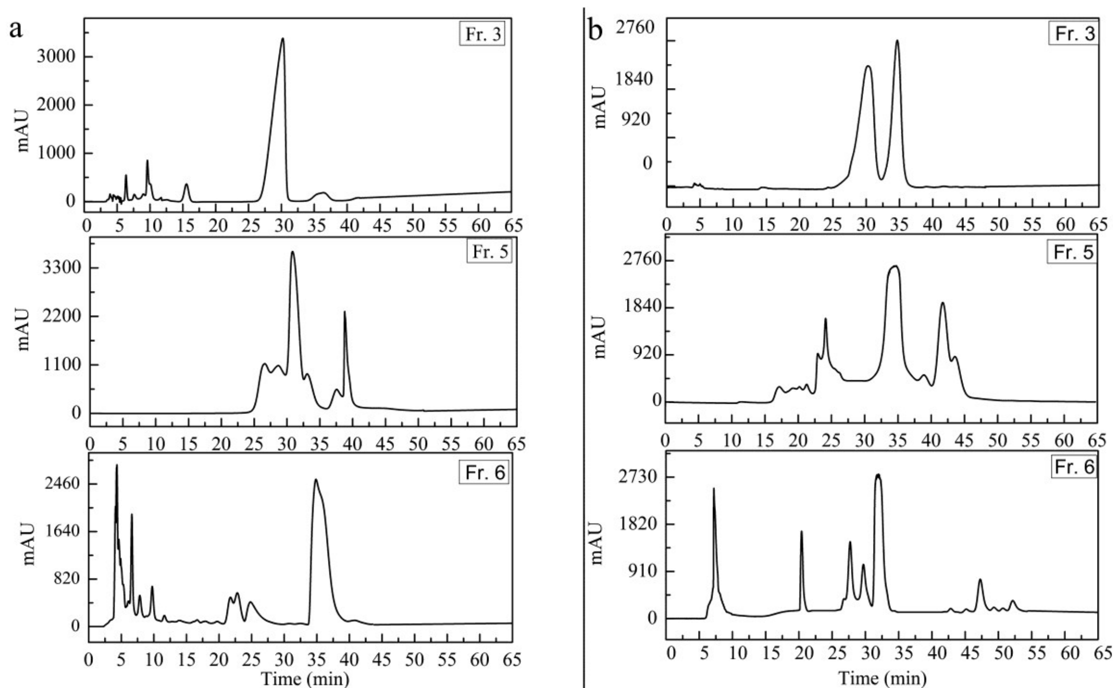
435 

436 **Figure 1. (a)** One-dimensional analysis chromatogram of the ethyl acetate extract of
437 *Euphorbia lathyris* on Agela bare silica column (4.6 × 250 mm, 10 μm, 100 Å). **(b)**
438 One-dimensional preparation chromatogram of the ethyl acetate extract of *Euphorbia*
439 *lathyris* on Agela bare silica column (150 × 250 mm, 10 μm, 100 Å).

440 

441 **Figure 2. (a)** Second-dimension analysis chromatogram of Fr. 3, Fr. 5 and Fr. 6 on
442 Agela bare silica column (4.6 × 250 mm, 10 μm, 100 Å). **(b)** Second-dimension
443 analysis chromatogram of Fr. 3, Fr. 5 and Fr. 6 on YMC amide column (4.6 ×250 mm,
444 10 μm, 100 Å).

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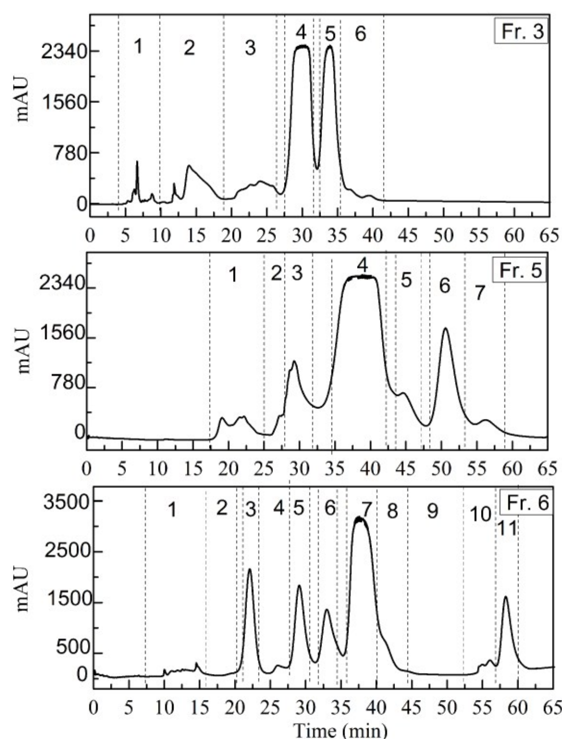

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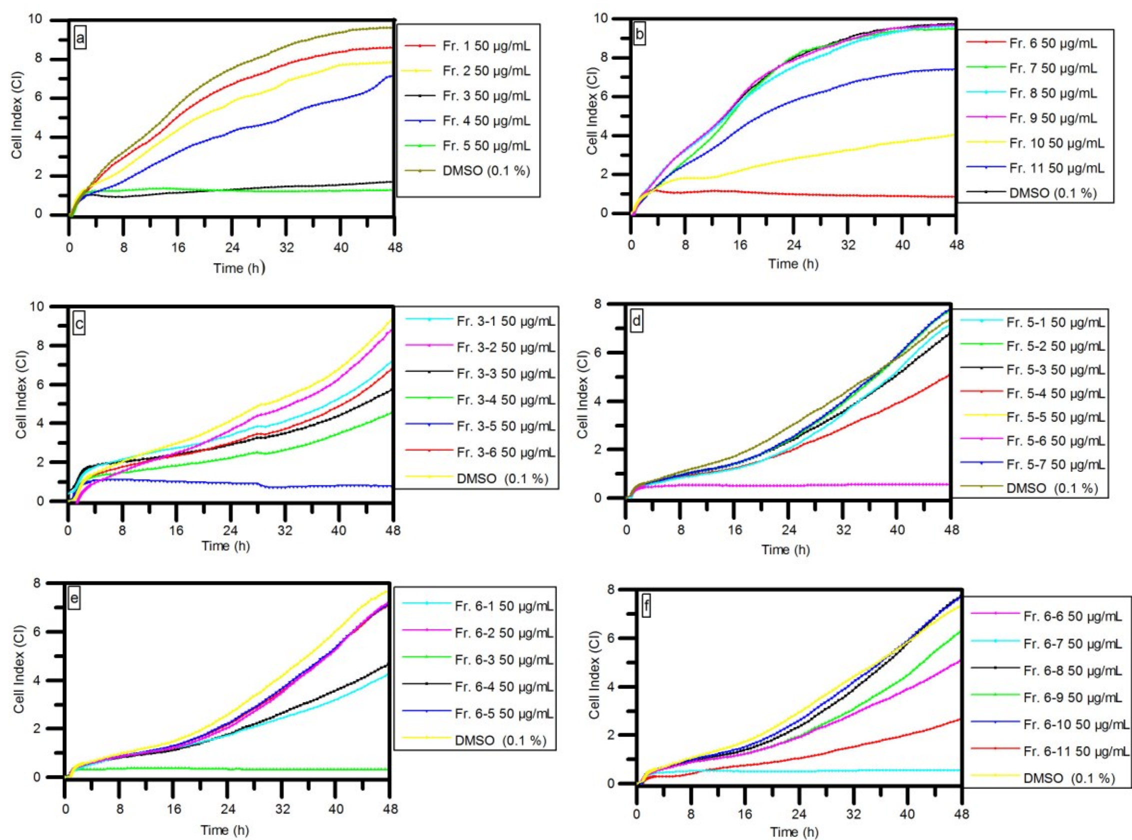
446 **Figure 3.** Second dimensional preparation chromatogram of Fr. 3, Fr. 5 and Fr. 6 on
447 YMC amide column (10 mm × 250 mm, 10 μm, 100 Å).



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449 **Figure 4.** Effect of fractions on the viability of A549 cells, determined by use of the

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iCELLigence RTCA system. Human lung cancer A549 cells at a density of 20,000 cells/well in E-Plate L8 were observed for 48 h. **(a)** Anti-lung cancer effect of Fractions 1–5. **(b)** Anti-lung cancer effect of Fractions 6–11. **(c)** Anti-lung cancer effect of fractions 3-1–3-6. **(d)** Anti-lung cancer effect of fractions 5-1–5-7. **(e)** Anti-lung cancer effect of fractions 6-1–6-5. **(f)** Anti-lung cancer effect of fractions 6-6–6-11.

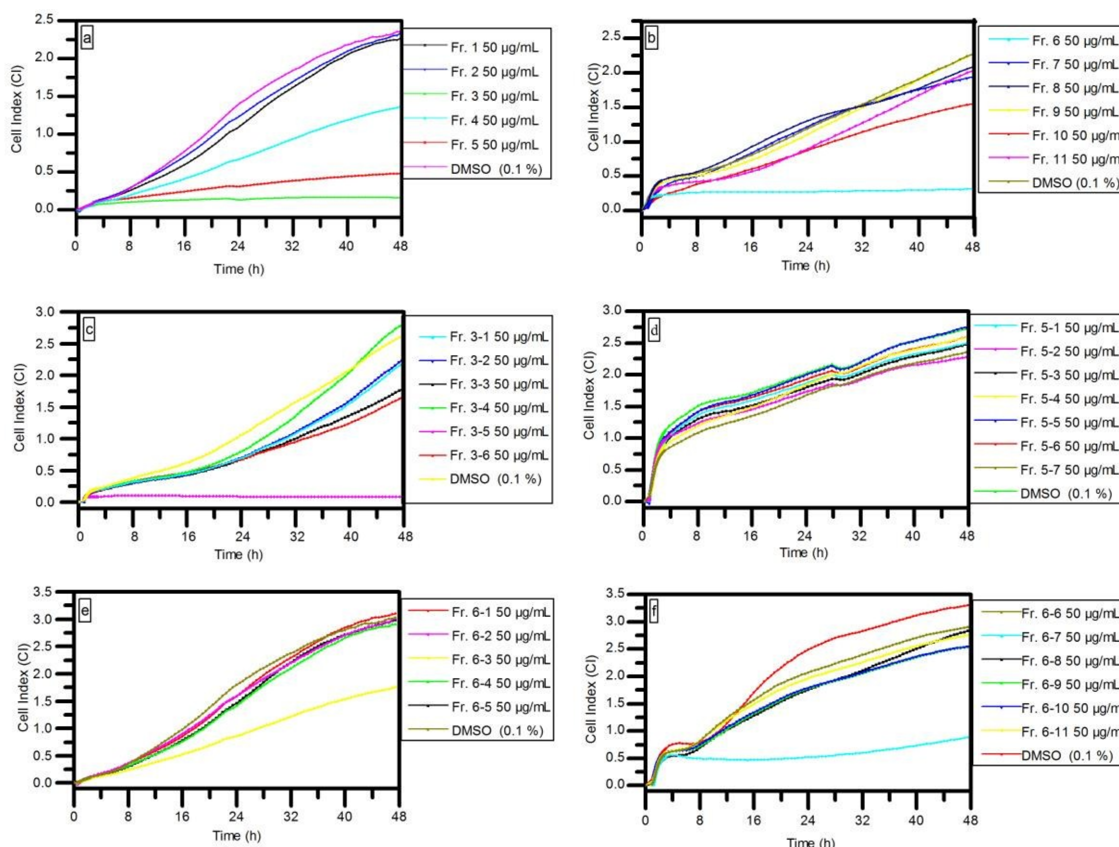

Figure 5. Effect of fractions on the viability of HEPG2 cells, determined by use of the iCELLigence RTCA system. Human liver cancer HEPG2 cells at a density of 40,000 cells/well in E-Plate L8 were observed for 48 h. **(a)** Anti-liver cancer effect of Fractions 1–5. **(b)** Anti-liver cancer effect of Fractions 6–11. **(c)** Anti-liver cancer effect of fractions 3-1–3-6. **(d)** Anti-liver cancer effect of fractions 5-1–5-7. **(e)** Anti-liver cancer effect of fractions 6-1–6-5. **(f)** Anti-liver cancer effect of fractions 6-6–6-11.

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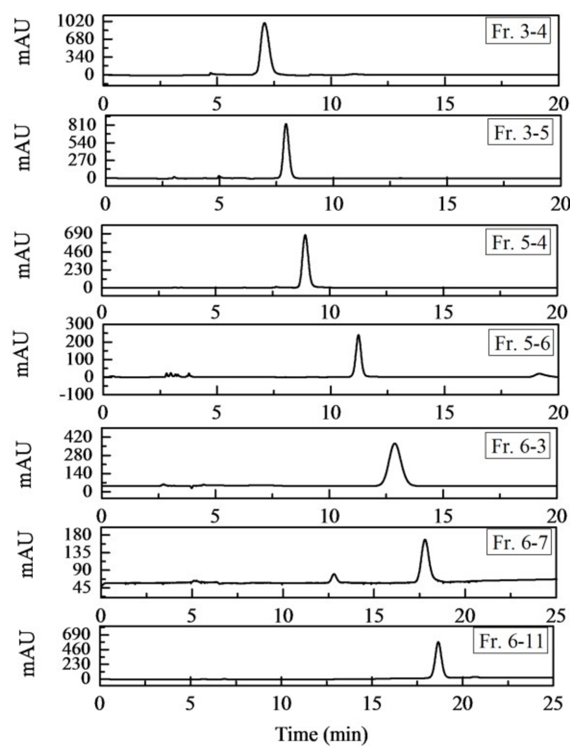

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465 **Figure 6.** The purity of isolated compounds was tested by HPLC using a YMC diol

466 column (4.6 × 250 mm, 5 μm, 100 Å).

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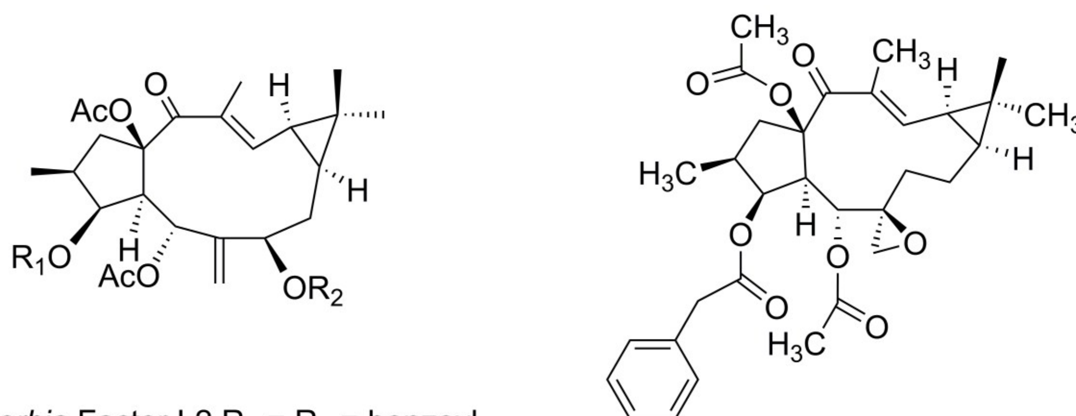
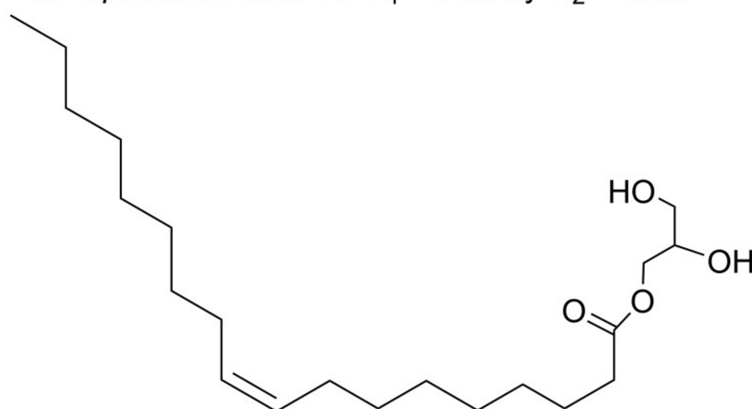
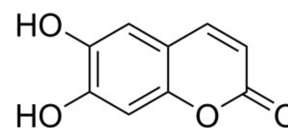


1. *Euphorbia* Factor L2 $R_1 = R_2$ = benzoyl
4. *Euphorbia* Factor L8 R_1 = ONic
5. *Euphorbia* Factor L3 R_1 =benzoy
6. *Euphorbia* Factor L9 R_1 = benzoy R_2 = ONic

2. *Euphorbia* Factor L1





3. Glyceryl monooleate

7. Esculetin

Figure 7. Chemical structures of bioactive compounds.

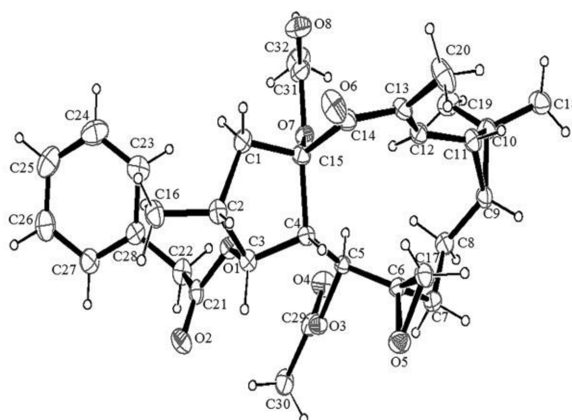


Figure 8. X-ray single-crystal diffraction structural drawing with displacement ellipsoids showing the perspective view of the moleculear conformation of compound 2, with the atom numbering scheme**.**

Supplementary

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4 474 **Table S1.** ^{13}C NMR spectroscopic data for compounds 1-6 in $CDCl_3$, compound 7 in
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6 475 DMSO (100 MHz)
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8 476 **Table S2.** ^{1}H NMR data for compounds 1-6 in $CDCl_3$, compound 7 in DMSO (400
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10 477 MHz)
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12 478 **Table S3.** Crystal data and structure refinement for compound 2
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14 479 **Table S4.** Selected Bond Lengths (Å) for compound 2
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16 480 **Table S5.** Selected Bond Angles (°) for compound 2
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18 481 **Figure S1.** U-shape plots of the retention factors of caffeic acids, k, versus the volume
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20 482 fraction of water, φ (water)
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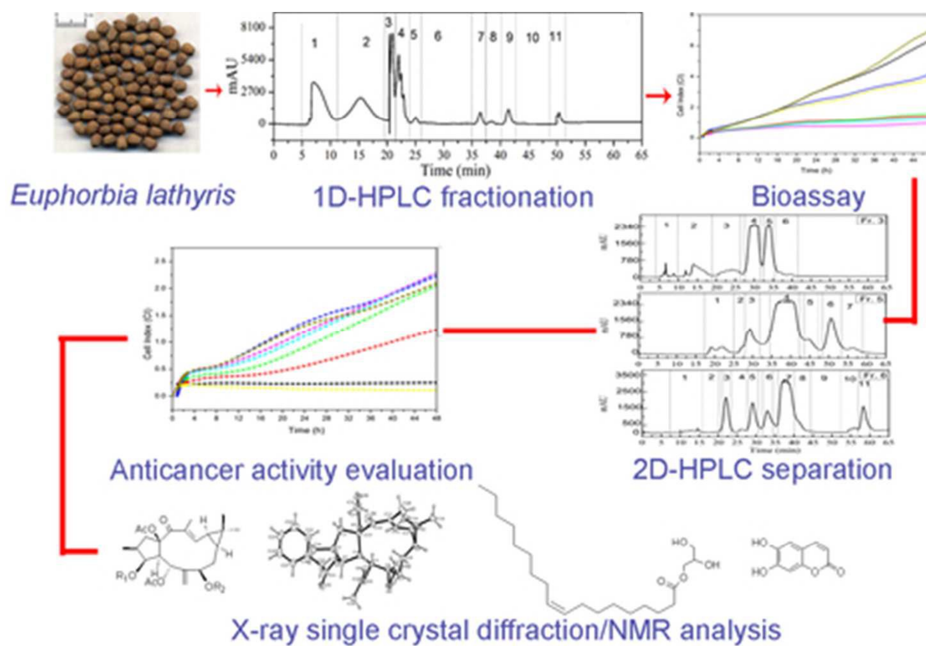

Bioactivity-guided isolation of anticancer compounds from Euphorbia lathyris
39x27mm (300 x 300 DPI)

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